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#1215375

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	Group Art Unit 1681
Van Beek	)	
	)	
Serial No. 09/753,381	)	Examiner: Irene Marx
Filed: January 2, 2001	)	Appeal No.: 2006-0151
For: Method for Improving the Activity	)	
Of Enzymes	)	

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Submission of Appendices

Applicant mistakenly omitted the Evidence Appendix and Related Proceedings Appendix required by 37 C.F.R. 41.37(c)(ix) and (x). These Appendices are enclosed.

Respectfully Submitted,

Date: 11/22/05

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	Group Art Unit 1681
Van Beek	)	
	)	
Serial No. 09/753,381	)	Examiner: Irene Marx
Filed: January 2, 2001	)	Appeal No.: 2006-0151
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RELATED PROCEEDINGS APPENDIX

None.

#1214836

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	Group Art Unit 1681
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Of Enzymes	)	

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EVIDENCE APPENDIX

	Document	Date Entered
1.	Sas, B., Peys, E. and Helsen, M. 1999. Efficient method for (lyso)phospholipids class separation by high-performance liquid chromatography using an evaporative light-scattering detector. J. Chromatography A, 864:1:179-182	1/02/2001 (filing date of application)
2.	United States Patent No. 6,068,997	1/02/2001 (filing date of application)

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Short communication

Efficient method for (lyso)phospholipid class separation by high-performance liquid chromatography using an evaporative light-scattering detector

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Abstract

A simple high-performance liquid chromatography method with evaporative light-scattering detection has been devised in order to separate and quantify the major phospholipid and lysophospholipid classes. HPLC analyses were performed with a diol–silica column and ternary gradient elution. Standard curves were drawn up for each of the (lyso)phospholipids involved.

Keywords: Lysophospholipids; Phospholipids; Lipids

1. Introduction

(Lyso)phospholipids are the subject of intensive study in medical science, pharmaceutics, cosmetics, food and feed industry. Their use as components in numerous industrial applications is well established. Most of these industrially used phospholipids are obtained as a by-product of the vegetable oil production and have a varying composition. Since variables responsible for efficacy in different applications are still quite unknown it is often not easy to asses the serviceableness of these crude products. The concept of quality therefore in many cases relates to the chemical composition or (lyso)phospholipid class distribution. A variety of methods have been published describing (lyso)phospholipid class separation utilising high-performance liquid chromatography. The separation of a number of major classes has been accomplished with a variety of columns (silica, diol–silica, amino–silica), eluent systems (isocratic as well as gradient) and detectors (phosphorus analyser, UV, radioactive flow, flame ionisation, fluorescence, evaporative light-scattering) [1–18] The reported number of (lyso)phospholipid classes separated ranges from four to seven. With the solvent system and elution scheme presented in this paper, eight (lyso)phospholipid classes can be separated and quantified simultaneously.

2. Experimental

2.1. Chemicals

Standard solutions of 10 mg ml^{-1} of phosphatidic

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acid (PA, egg origin), phosphatidylcholine (PC, soybean origin), phosphatidylethanolamine (PE, egg origin), phosphatidylinositol (PI, soybean origin), phoshatidylglycerol (PG, egg origin), lysophosphatidic acid (LPA, synthetic, 1-palmitoyl), lysophosphatidylcholine (LPC, plant origin), lysophosphatidylethanolamine (LPE, synthetic, 1-oleolyl), and lysophosphatidylinositol (LPI, liver origin) in chloroform were purchased from Avanti Polar Lipids (AL, USA). All materials were more than 99% of the compound stated. Triethylamine (*purum*) was purchased from Fluka (Buchs, Switzerland). All other solvents were purchased from Acros Organics (Geel, Belgium) and were HPLC-grade except for acetic acid which was analytical-reagent grade.

2.2. Equipment

HPLC analysis was performed with a Spectra-Physics system (Spectra-Physics, San José, CA, USA) equipped with an SP 8880 autosampler, an SP 8800 ternary pump and a Chromjet integrator. The Spectra System PC1000 software (Spectra-Physics Analytical, Fremont, CA, USA) was used for instrument control, data acquisition and data analysis. Detection was carried out using a Varex MKIII evaporative light scattering detector (Alltech, Deerfield, IL, USA).

2.3. HPLC analysis

A YMC-Pack Diol-NP column (S 5 μm, 120 A, 250×4.6 mm I.D., YMC, Japan) was used at an ambient temperature of about 21°C. The injector valve of the autosampler was fitted with a 20 μl loop. The separation was performed by gradient elution using solvent systems (A) *n*-hexane, (B) methanol–acetic acid–triethylamine (765.7:15.31: 12.9, w/w) and (C) acetone–acetic acid–triethylamine (765.4:15.31:12.9, w/w). Eluents were continuously flushed with helium. Total run time was 46 min. The gradient started with the ratio of 7% A, 0% B and 93% C. Throughout the complete gradient cycle, A stayed at 7%, %B increased gradually while %C decreased to the same extent. B was held at 50% during 4 min, then decreased linearly to 0% over a period of 10 min and stayed there for another 4 min before the next run started (Table 1). The first 32 min period of the elution scheme was designed to separate the (lyso)phospholipid components, and the final 14 min regenerated the column prior to injection of the next sample. The flow-rate was 1 ml/min. The N_2 flow-rate and the drift tube temperature of the detector were maintained at 1.9 l/min and at 85°C respectively throughout all analyses.

Table 1
High-performance liquid chromatography gradient program

Time (min)	A (%)	B (%)	C (%)
0	7	0	93
6	7	4	89
10	7	6	87
11	7	11	82
16.5	7	15	78
18.5	7	25	68
22	7	30	63
26	7	40	53
28	7	50	43
32	7	50	43
42	7	0	93
46	7	0	93

2.4. Data analysis

Calibration curves were obtained for individual (lyso)phospholipids. This was done at quantities injected between 1 and 8 μg (1, 1.6, 3.2, 4.8, 6.4 and 8 μg) for PA and LPC, between 1.6 and 8 μg (1.6, 3.2, 4.8, 6.4 and 8 μg) for PI, between 2 and 10 μg (2, 4, 6, 8 and 10 μg) for PC, PE, PG, LPA, LPE and LPI. Each quantity was injected four times. Standard calibration curves were obtained from least-squares linear regression analysis. Area units in millions (mV s) were plotted as a function of the quantity injected (μg). The linearity of the method was statistically tested. Retention time stability was calculated for all classes using the retention times off all samples injected to draw their calibration curves. A mixture of the above (lyso)phospholipid standards was injected. The amounts of PA, PC, PE, PG, PI, LPA

LPC, LPE, and LPI injected were 3, 7, 6, 2, 8, 6, 5, 5 and 5 μg, respectively.

3. Results and discussion

The response of the evaporative light scattering detector is non-linear but within a limited range it can be described by a linear model. The method showed linearity for each (lyso)phospholipid class over the range stated above except for LPA and LPE were linearity was limited to the 4–10 μg ml^{-1} range. Correlation coefficients (R) obtained from the plot of experimental values as a function of theoretical values were always above 0.997 for the range of linearity (Table 2).

With the amount of phospholipid injected onto the column in this experiment being between 1 and 10 μg, these results disagree with Van der Meeren et al. [15] who reported a non-linear response of the evaporative light scattering detector from 0 to 50 μg of phospholipids. This may be not a real disagreement but could be attributed to the different range size considered. Christie [16] reported that linear response of the evaporative light scattering detector dropped off drastically below 10 μg. Present results confirm these of Breton et al. [17] who obtained a linear response for amounts of 5–40 μg of phospholipids and those of Mounts et al. [18].

Baseline was stable and retention times of all (lyso)phospholipid species were well reproducible with standard deviations ranging from 0.04 for PA, eluting at the beginning of a run, to 0.21 for LPC, eluting close to the end of the separation part of a run. The chromatogram resulting from injecting a mixture of the (lyso)phospholipid standards used is shown in Fig. 1. Since the retention times for PA and PG are not that wide apart, peak resolution may sometimes be insufficient when both are present. The possibility of using this application for quantification of (lyso)phospholipid classes present in crude vegetable oil lecithin and in hydrolysed products made from it is presently being explored.

Table 2
Data analysis of the method for the tested (lyso)phospholipid classes

Class	Linear regression equation $y = ax + b$[a]			Correlation coefficient (R)	Retention time (SD)
	Slope, a (SE)	Intercept, b (SE)	n		
PA	1.288 (0.013)	−0.896 (0.061)	24	>0.998	6.19 (0.04)
PC	1.367 (0.008)	−1.013 (0.053)	20	>0.999	20.18 (0.20)
PE	1.432 (0.026)	−0.525 (0.178)	20	>0.997	7.58 (0.08)
PI	1.302 (0.022)	−1.089 (0.118)	20	>0.997	15.09 (0.21)
PG	1.451 (0.006)	−1.382 (0.037)	20	>0.999	6.48 (0.04)
LPA	0.976 (0.009)	−1.660 (0.069)	16	>0.999	10.76 (0.09)
LPC	1.072 (0.009)	−0.459 (0.045)	24	>0.999	23.71 (0.21)
LPE	1.201 (0.015)	−2.318 (0.112)	16	>0.998	11.81 (0.10)
LPI	1.543 (0.024)	−1.838 (0.160)	20	>0.998	18.26 (0.13)

[a] x = μg injected; y = peak area in 10^6 mV s.

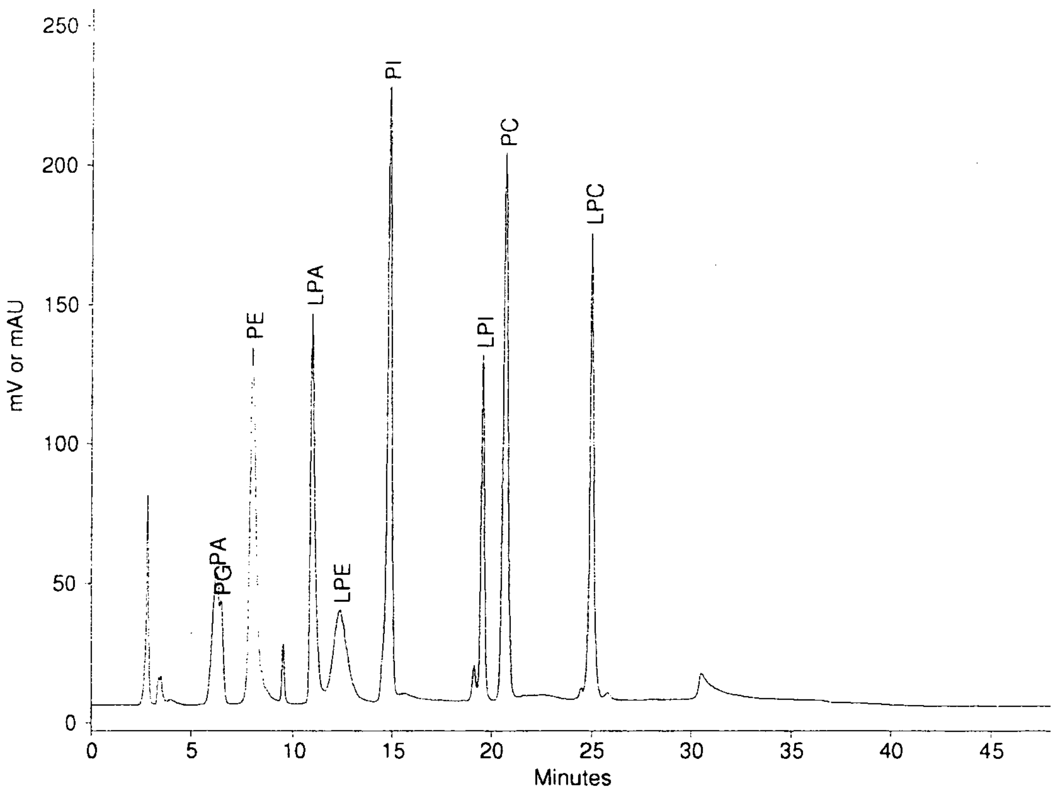

Fig. 1. Separation of (lyso)phospholipids by HPLC on a YMC-Pack Diol-NP column (250×4.6 mm, 5 μm, 120 A), evaporative ligi scattering detection (other conditions see text). Phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE phosphatidylglycerol (PG), phosphatidylinositol (PI), lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysopho phatidylethanolamine (LPE), lysophosphatidylinositol (LPI).

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